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Mash1 efficiently reprograms rat astrocytes into neurons

Daofang Ding[1,2], Leqin Xu[1,2], Hao Xu[1,2], Xiaofeng Li[1,2], Qianqian Liang[1,2], Yongjian Zhao[1,2], Yongjun Wang[1,2]

1 Institute of Spine, Shanghai University of Traditional Chinese Medicine, Shanghai, China
2 Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

Daofang Ding and Leqin Xu contributed equally to this paper.

Corresponding author:
Yongjun Wang, M.D., Ph.D., Institute of Spine, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China; Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China, yjwang88@hotmail.com.

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Abstract

To date, it remains poorly understood whether astrocytes can be easily reprogrammed into neurons. Mash1 and Brn2 have been previously shown to cooperate to reprogram fibroblasts into neurons. In this study, we examined astrocytes from 2-month-old Sprague-Dawley rats, and found that Brn2 was expressed, but Mash1 was not detectable. Thus, we hypothesized that Mash1 alone could be used to reprogram astrocytes into neurons. We transfected a recombinant MSCV-MASH1 plasmid into astrocytes for 72 hours, and saw that all cells expressed Mash1. One week later, we observed the changes in morphology of astrocytes, which showed typical neuronal characteristics. Moreover, β-tubulin expression levels were significantly higher in astrocytes expressing Mash1 than in control cells. These results indicate that Mash1 alone can reprogram astrocytes into neurons.

Key Words: nerve regeneration; Mash-1; Brn2; β-tubulin; reprogram; plasmid; retrovirus; astrocytes; neurons; the National Basic Research Program of China (973 Program); neural regeneration

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Introduction

Following spinal cord injury, the poor regeneration of neurons, with increased gliogenesis and bone morphogenetic protein expression leads to defects in motor function[1-2]. In recent years, the transplantation of fetal neurons has shown some potential for the effective treatment of patients with neurological disorders[3]. Furthermore, neural stem cells have been reported to show good potential for treatment of spinal cord injury[4]. However, there are still a number of limitations associated with cell transplantation, including efficient methods of obtaining sufficient sources of cells, immunological rejection and ethical issues. Nowadays, several lines of evidence suggest that neurons obtained from other cell types could address all these limitations associated with cell transplantation[5-13].

Cellular reprogramming is a new and rapidly developing technology in which somatic cells can be induced into pluripotent stem cells or other somatic cell types simply by the expression of specific genes[5-13].

Human embryonic stem cells and induced pluripotent stem cells can be differentiated into neuronal cells with a high efficiency and purity in a short time by forced expression of a single transcription factor[5]. Recently, fibroblasts were used as a direct source for induced neuronal cells[6-8]. Apart from the direct induction of fibroblasts into neuronal cells, some induced neuronal cells have also been obtained from the intermediate generation of a partially reprogrammed pluripotent state, including induced neural stem cells derived from fibroblasts by forced expression of reprogramming genes[9-11]. Furthermore, terminally differentiated hepatocytes were reported to have been reprogrammed into neuronal cells[12]. Astrocytes, the most abundant cells in the central nervous system, are an ideal cell population for the direct generation of neurons[13]. With the development of these methods, we may find a new treatment for Parkinson's disease[14-17].

Changes of growth condition also help promote the reprogramming of cells into neurons, and three-dimensional spheres could endow somatic cells with the stemness of neural stem cells[18]. Hypoxic conditions during the initial reprogramming stages significantly enhance the efficiency of inducing human fibroblasts into neuronal cells[19]. Mouse fibroblast cells were transdifferentiated into an intermediate state characterized by spherical cell aggregates, somewhere between mouse embryonic stem cells and mouse fibroblasts, using specialized medium containing lipid-rich albumin. Such spheres were able to differentiate into ectodermal germ layers following retinoic acid treatment[20]. microRNAs, such as miR9* and miR-124, participated in the transdifferentiation of fibroblasts into neuronal cells in the presence of other neurogenic transcription factors[21-22]. Small molecule inhibitors of glycogen synthase kinase-3β and Smad signaling can also promote increased yields and purity[23].

During nervous system development, neuronal differentiation is governed by a series of transcription factors, especially the basic helix-loop-helix transcription factor[24-26]. These transcription factors play different roles in instructing the generation of different neuronal subtypes. Specifically, Mash1 plays an important role in brain development, in which the functional loss of Mash1 results in severe defects in basal ganglia neurons and cortical interneurons in the telencephalon[27-30].

A number of studies have demonstrated that Mash1 was efficient for differentiating stem cells or reprogramming distinct cell types into neurons. Mash1 transfection into embryonic stem cells and their transplantation are powerful tools for the treatment of hemiplegia in the mouse model, where they have been shown to improve motor function[31-32]. They have also been shown to improve motor function in the mouse model of spinal cord injury[33]. Neurons can be generated from the embryonic carcinoma cell line P19[34]. From a perspective of regenerative medicine, obtaining purified neurons from embryonic carcinoma cells and embryonic stem cells may be difficult. Not all embryonic stem cells or embryonic carcinoma cells can differentiate into neurons after the ectopic expression of Mash1, and the formation of tumors is not easily controlled during this process. To avoid these obstacles, alternative, safer cell sources must be obtained.

Other groups have shown the induction of expression of a combination of neurogenic transcription factors, including Mash1, Brn2 and Mtyl1 or by Mash1, Nurr1 and Lmx1a, to promote neurogenic reprogramming of murine or human fibroblasts *in vitro*[35-36]. In these studies, it appears that Mash1 is indispensable in reprogramming fibroblasts into neurons. Primary astrocytes can also be induced into glutamatergic neurons in the presence of Neurog2 or GABAergic neurons by Dlx2 or Dlx2 plus Mash1[37-39].

Based on the above findings, we hypothesized that Mash1 alone could reprogram rat astrocytes into neurons efficiently. To test this hypothesis, Brn2 levels were examined to determine whether it is endogenously expressed in rat astrocytes. Murine stem cell virus (MSCV)-Mash1 recombinant plasmid was constructed and transfected into GP2-293t cells to produce retrovirus. Rat primary astrocytes were infected with Mash1 retroviruses, which results in successful reprogramming of astrocytes into neuron-like cells when maintained in neuronal medium. The neuronal marker gene β-tubulin was detected by immunostaining to verify the phenotype of neurons.

Results

Construction of recombinant plasmid

To stably express Mash1 in astrocytes, we chose the MSCV viral vector as the expression system. The length of Mash1 was 702 bp. Mash1 was inserted between *Xho* I and *Bgl* II restriction sites of the T-vector to obtain T-Mash1. Mash1 was digested from T-Mash1 and ligated to MSCV to obtain the recombinant plasmid MSCV-Mash1 (Figure 1).

Culture and identification of primary astrocytes

Gray matter is a neuronal cell population that includes astrocytes and oligodendrocytes. In our experiment, oligodendrocyte precursor cells were excluded from the cell population by severely shaking the flask. Astrocytes were passaged and cultured on glass coverslips (Figure 2A). The expression of glial fibrillary acidic protein, an astrocyte marker, was detected by immunostaining after 1 week of culture. A vast majority of cells were positive for glial fibrillary acidic protein (Figure 2B–D).

Endogenous expression of Brn2 in astrocytes

We further investigated if other neural developmental transcription factors were expressed in these cells after astrocytes were cultured and identified. Strikingly, we found that Brn2, which plays key roles in the reprogramming of astrocytes into neurons, was also detected in astrocytes. Almost all astrocytes strongly expressed Brn2 in the nucleus (Figure 3).

Mash1 overexpression induced neuronal specification of astrocytes

Mash-1 virus was produced in GP2-293t cells with the helper plasmid PMD2.0G. Astrocytes were infected by Mash1 retroviruses and protein expression was validated by immunostaining 72 hours post-infection. All cells were positive for Mash1 (Figure 4A). The morphology of astrocytes was not typical; most became elongated after ectopic Mash1 expression. Both Mash1-infected astrocytes and empty vector-infected astrocytes were maintained in neuronal medium and allowed to differentiate for different time periods. We found subtle morphological changes in Mash1-infected astrocytes at days 3 and 5 (data not shown), but at day 7, neuronal axons began to develop and their appearance became more similar to neurons (Figure 4B).

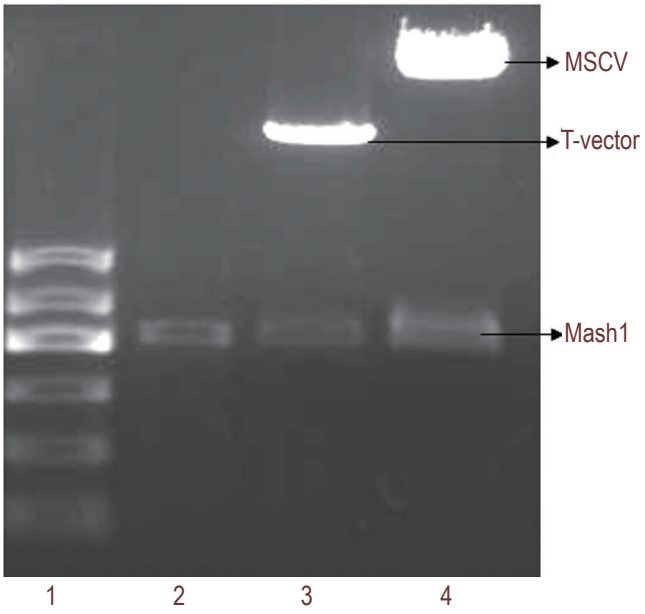

Figure 1 Construction of the murine stem cell virus (MSCV)-Mash1 recombinant plasmid (polymerase chain reaction).
Lane 1: DNA marker; lane 2: reverse transcription-polymerase chain reaction analysis of Mash-1; the length is 702 bps. Lanes 3 and 4: restriction enzyme analysis of T-Mash1 and MSCV-Mash1, respectively.

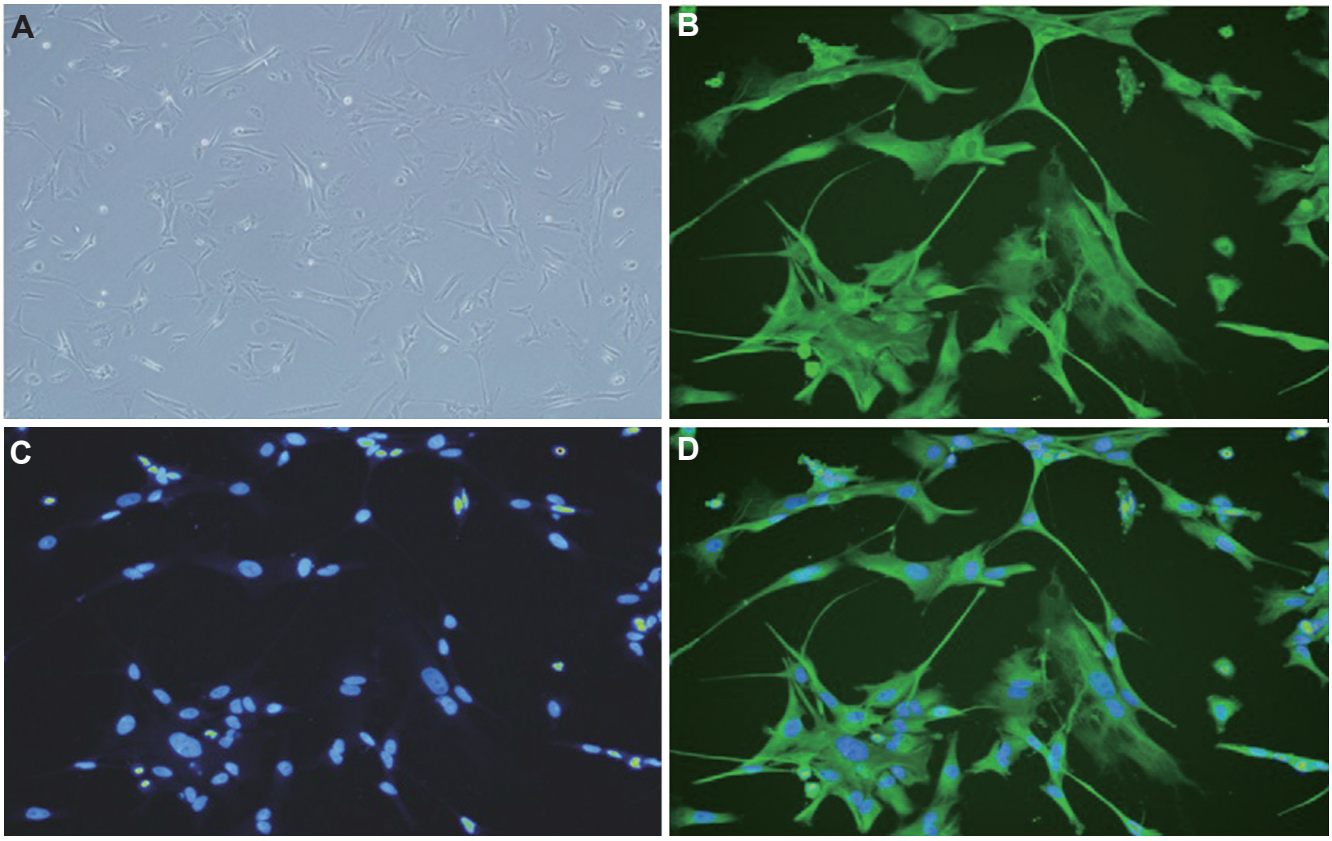

Figure 2 Identification of glial fibrillary acidic protein expression in primary astrocytes.
(A) Phase-contrast of purified primary astrocytes (× 100, light microscope). (B–D) Detection of glial fibrillary acidic protein expression in cultured primary astrocytes by immunostaining 1 week later (× 200, inverted fluorescence microscope). Astrocytes are labeled in green with glial fibrillary acidic protein antibody (B), and 4′,6-diamidino-2-phenylindole (DAPI) stained cell nuclei in blue (C). The merged picture (D) shows the cells positive for glial fibrillary acidic protein.

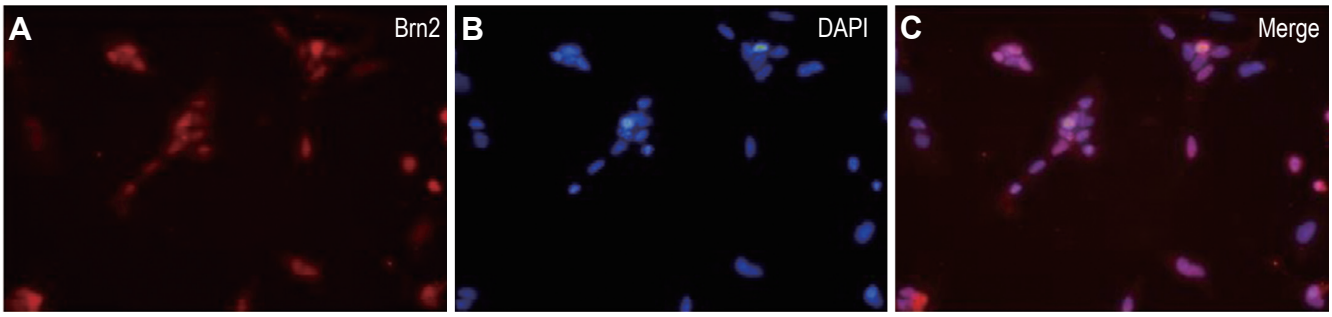

Figure 3 Analysis of Brn2 expression in astrocytes by immunostaining (× 200, inverted fluorescence microscope).
Astrocytes are labeled in red with Brn2 antibody (A). 4′,6-Diamidino-2-phenylindole (DAPI) marks all cell nuclei in blue (B). The merged picture (C) shows the cells positive for Brn2.

β-Tubulin expression increased in Mash1-infected astrocytes

Except for the typical morphological changes into neuronal-like cells, protein expression of β-tubulin was also analyzed to confirm reprogramming. The results of immunofluorescence showed that Mash1-infected cells were positively stained for β-tubulin expression and the expression level in the MSCV empty vector-infected cells was much weaker (Figure 5A). Furthermore, the results of western blot assay were in accordance with the results of immunostaining (Figure 5B).

Discussion

Mash1 is an important regulator in determining whether a cell remains a progenitor or differentiates into a terminal cell type, by acting as an inhibitor of Notch signaling, thus balancing progenitor and differentiation states[40]. It has also been shown to be a critical component in the cocktail (along with Pou3f2 and Mytl1 or with Dlx2) required for directly reprogramming fibroblasts or astrocytes into neurons.

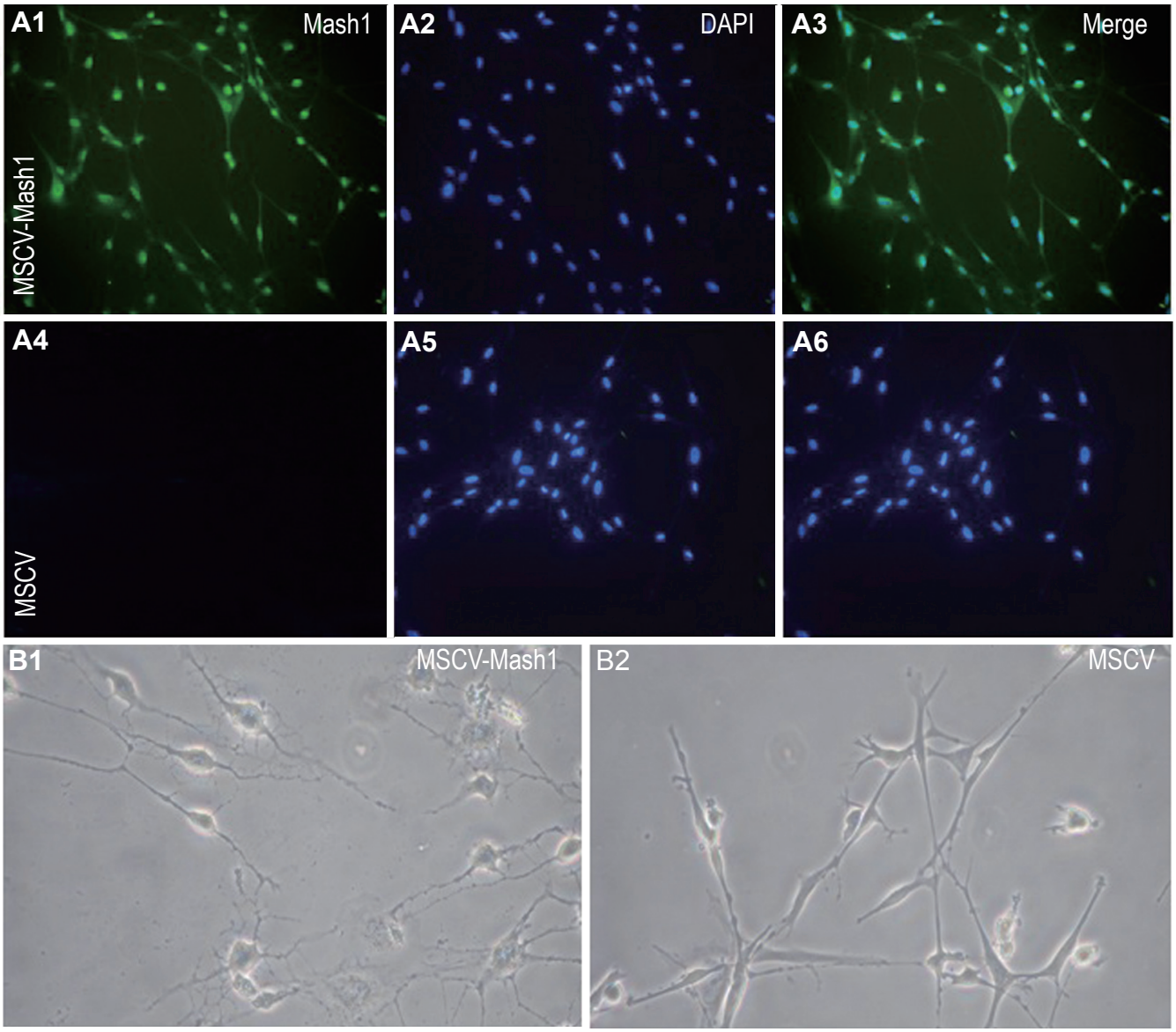

Figure 4 Morphological changes of astrocytes induced by Mash1 overexpression (× 200).
(A1–A6) Identification of Mash1 expression in astrocytes infected by murine stem cell virus (MSCV)-Mash1 and MSCV retroviruses 72 hours post-infection. All cells were positive for Mash1. Mash1-positive cells are in green; 4′,6-diamidino-2-phenylindole (DAPI) marked all cell nuclei in blue under an inverted fluorescence microscope. (B1, B2) Astrocytes were infected by MSCV or MSCV-Mash1 and cultured in neuronal medium for 7 days. Neuronal axons began to develop in astrocytes infected by Mash1 and their appearance became neuronal-like.

Whether the reprogramming effect of Mash1 on fibroblasts or astrocytes from mouse and human is universal to cells derived from other animals, such as rats, requires further study. Previous studies have shown that other transcription factors besides Mash1 may be required to reprogram somatic cells into neurons. In these studies, transcription factors were delivered to the host cells in the form of viruses. This method of transgene expression has been shown to be efficient in obtaining reprogrammed cells. However, a major limitation of this technology is that viruses can integrate into the genome and induce malignant transformation due to the spontaneous reactivation of viral transgenes[41-42]. Decreasing the number of the transcription factors required for reprogramming could reduce the risk of tumor formation. Based on a previous study, we aimed at determining an easier process of reprogramming through the endoge-nous expression of important transcription factors.

We chose astrocytes as target cells for this study as they are from the same germ layer as neurons. To investigate whether Mash1 alone could reprogram rat astrocytes into neurons, the full-length Mash1 gene from rat brain tissue was amplified with specific primers. To stably express Mash1 in astrocytes, Mash1 was cloned into MSCV and the recombinant plasmid was transfected into the GP2-293t cell line with PMD2.0G to produce retrovirus. We isolated primary astrocytes from grey matter tissue of 2-month-old rat brain and propagated the cells in astrocytic medium. Oligodendrocytes were removed by severely shaking the flask according to their different adhesion capacities to dish bottom. Purified astrocytes could be obtained by this method. Further immunoflu-

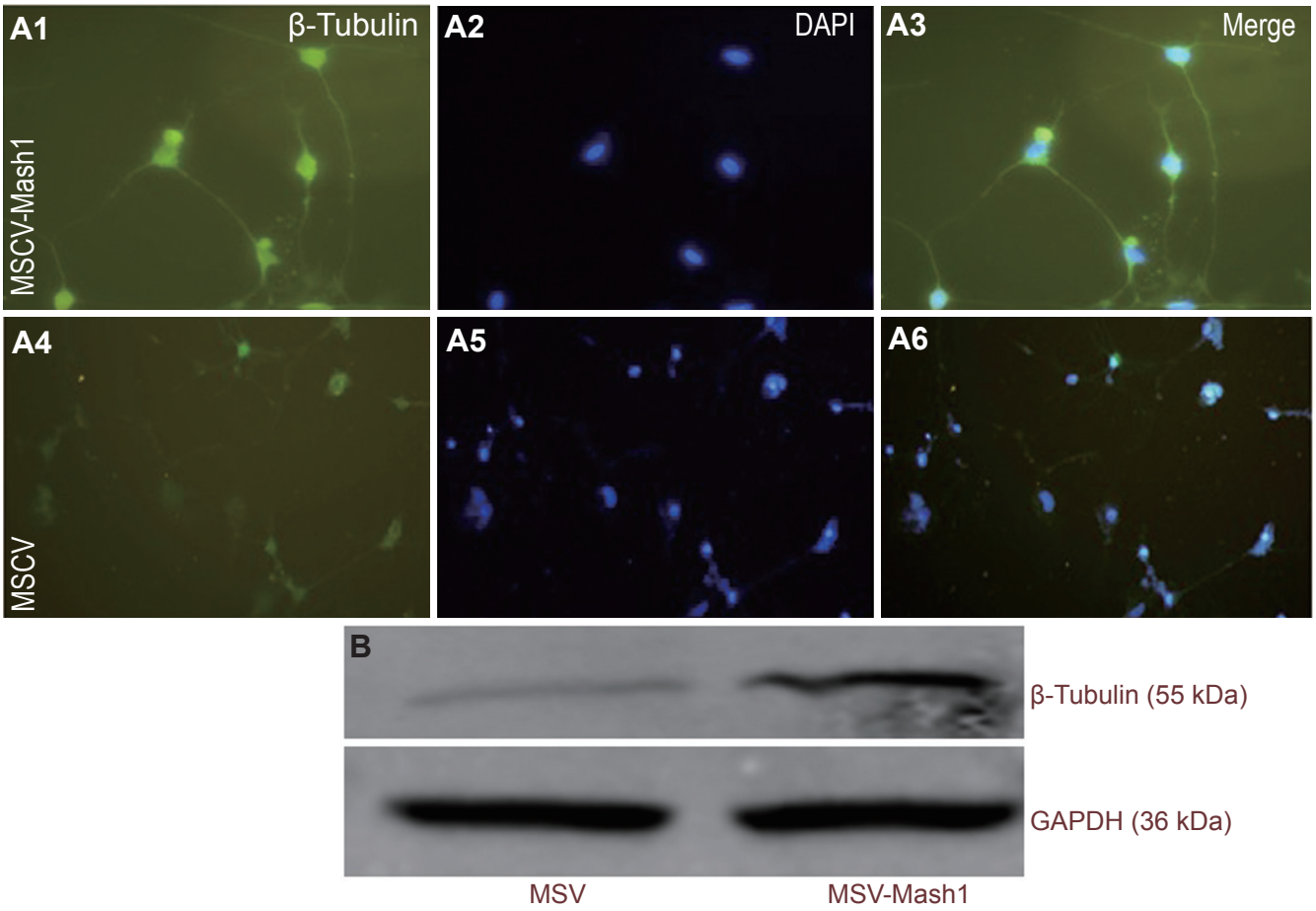

Figure 5 β-Tubulin expression in neurons derived from astrocytes.
(A1–A6) Immunostaining analysis of β-tubulin in astrocytes infected with murine stem cell virus (MSCV)-Mash1 and MSCV (200 ×, inverted fluorescence microscope). β-Tubulin-positive cells are in green; 4′,6-diamidino-2-phenylindole (DAPI) marked all cell nuclei in blue. (B) The protein expression levels of β-tubulin in astrocytes infected with MSCV-Mash1 and MSCV were analyzed by western blot assay 7 days post-infection. Mash1-infected cells were positive for β-tubulin expression.

orescence results confirmed the expression of glial fibrillary acidic protein and the purification of astrocytes. Almost 99% of cells were positive for glial fibrillary acidic protein.

Purified astrocytes were infected with Mash1 retrovirus and its expression was quantified 3 days post-infection. The results showed that all astrocytes were infected with the retrovirus and the expression of Mash1 was localized to the nucleus, which further confirmed Mash1 as a transcription factor. Furthermore, morphological changes of astrocytes infected by Mash1 could be identified compared with control cells, even from the third day. The astrocytes infected by Mash1 became elongated and neuronal axons began to appear.

It was reported that Mash-1 and Brn2 could cooperate to reprogram fibroblasts into neurons in neuronal medium[35]. Another study also showed that Mash1 and Dlx2 were efficient enough to induce the reprogramming of astrocytes into neurons. From previous studies, it could be deduced that Brn2 and Dlx2 are much more important than other transcription factors in reprogramming cells into neurons. This study reported for the first time that rat astrocytes could be easily reprogrammed into neurons with Mash1 alone. In previous studies, efficient reprogramming required a combination of several transcription factors. Thus, we hypothesized that other important transcription factors, such as Brn2 and Dlx2, must be endogenously expressed in astrocytes. We examined whether these proteins were expressed in astrocytes by cell immunofluorescence and found that they were positive for Brn2, but not for Dlx2 (data not shown).

Changes in the morphology of astrocytes infected by MSCV-Mash1 were noticeable by day 3. Neuron-like cells appeared from Mash1-infected cells after 7 days. Furthermore, immunostaining and western blot analysis revealed that Mash1 overexpression induced high-level expression of the neuronal marker β-tubulin in rat astrocytes.

Overexpression of Mash1 in neural stem cells causes rapid differentiation of transduced cells into functional neurons[43-44]. This study found that the high endogenous expression of Brn2 in astrocytes could result in efficient Mash1 reprogramming within 1 week. This finding could provide a useful way of obtaining neurons in a short time frame, and provide a basis for further study in the repair of spinal cord injury. Our primary astrocytes were isolated from adult 2-month-old Sprague-Dawley rats, which differ from some other studies; however, Mash1 has been shown to be universal in different species and somatic cells from different development stages.

Recent reports indicate that Mash1 is highly expressed in malignant tumors with neural or NE phenotype, such as neuroblastoma[45-47] and lung carcinoma[48-53]. In our study, these astrocytes do not pass a stem cell intermediate; direct neural conversion has the potential to be performed *in vivo*, which eliminates the risk for tumor formation. We did not investigate abnormal proliferation among Mash1-infected astrocytes, which may be a signal of malignant transformation, but we cannot rule out the possibility of this occurring. Revealing the complicated mechanism of the reprogramming process of astrocytes into neurons by Mash1 is essential and requires further investigation.

Materials and Methods

Design

A cellular, molecular, controlled study.

Time and setting

Experiments were performed at the Institute of Spine, Shanghai University of Traditional Chinese Medicine, China from March 2010 to July 2011.

Materials

Two-month-old male specific pathogen-free Sprague-Dawley rats were provided by Shanghai Xi-pu'Er-Bikai Co., Ltd., Shanghai, China, License No. SCXK (Hu) 2008-0016. The protocols for the animal experiments were conducted with the approval of the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine in China.

Methods

Cell culture

Sprague-Dawley rats were intraperitoneally anesthetized with 50 mg/kg sodium pentobarbital. After removal of the meninges, gray matter from the cerebral cortex was cut into small pieces and a single cell suspension was obtained by mechanical trituration. Subsequently, cells were centrifuged for 5 minutes at 1,000 r/min, resuspended and seeded in astrocytic medium consisting of 89% Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Big Cabin, OK, USA), 5% fetal calf serum (Biowest, Val de Loire, France), 5% horse serum (Biowest), 1% penicillin/streptomycin (Biowest). Contaminating oligodendrocyte precursor cells were removed by shaking the culture flasks several times. Cells were passaged and seeded on glass coverslips at a density of 60,000 cells per coverslip in the same medium.

Construction of recombinant plasmid and retrovirus production

Total RNA was extracted from rat brain tissues with Trizol (Cat. No. 15596-026; Invitrogen, Porirua, New Zealand, USA); RNA was reverse-transcribed into cDNA with reverse transcriptase (D2620, TaKaRa, Dalian, Liaoning Province, China). The primers for amplifying Mash-1 were as follows: 5′-ccgctcgaggaaccagttggtaaagtc-3′ (reverse) and 5′-aatagatctatggagagctctggcaag-3′ (forward). The PCR product was analyzed by 1% agarose gel electrophoresis with DNA Marker II (MD102-01; Tiangen, Beijing, China) as molecular weight standard and the target band was 702 bp. The purified PCR product was ligated into the recombinant cloning vector plasmid T-Mash1 (pGEM®-T Easy; Promega, WI, USA) with T4 ligase (#1231; Fermentas, Burlington, Canada). The recombinant plasmid T-Mash1 was sequenced to ensure that no mutation had been introduced. Mash1 cDNA from T-vector was inserted between *Bgl* II and *Xho* II restriction sites of MSCV retrovirus vector (Clonetech, Mountain View, CA, USA), under the control of Moloney Murine Leukemia Virus promoter.

Recombinant retrovirus particles were generated by transient transfection of GP2-293t cells using lipofectamine 2000 (Cat. No. 11668-019; Invitrogen, Carlsbad, CA, USA). Cells were cotransfected with MSCV-Mash1 and PMD2.0G (Addgene, Cambridge, MA, USA). The medium was replaced 4 hours after transfection and collected 48 hours later. Supernatants were passed through a 0.45-μm filter.

Retroviral transduction

Retroviral transduction of astrocytes was performed 24 hours after seeding on glass coverslips. For the control group, astrocytes were infected with viral particles from MSCV empty vector. For the experimental group, astrocytes were infected with MSCV-Mash1 viral particles. The experimental and control groups were infected and cultured in neuronal medium for 3 or 7 days. Day 3 and day 7 cells were used to detect Mash1 and β-tubulin expression, respectively, by immunocytochemistry.

For neuronal culture, at 24 hours after retroviral transduction, the astrocytic medium was removed from the cells and neuronal medium was added to infect astrocytes. Neuronal medium contained both 2% B27 (Gibco) and 98% DMEM/F-12.

Immunocytochemistry

Briefly, cultures including Mash1-infected astrocytes and MSCV empty vector-infected astrocytes were fixed in 4% paraformaldehyde in PBS (Biowest, Val de Loire, France) for 15 minutes at room temperature. Cells were first pretreated with 0.1% Triton X-100 in PBS for 30 minutes, followed by incubation in 2% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 minutes. Primary antibodies were incubated on specimens overnight at 4°C in 2% bovine serum albumin and 0.1 % Triton X-100 in PBS. The following primary antibodies were used: monoclonal mouse anti-glial fibrillary acidic protein (1:500; ab10062; Abcam, Cambridge, UK), polyclonal goat anti-Brn2 (1:200; SC-6029; Santacruz, Dallas, TX, USA), monoclonal mouse anti-β-tubulin (1:500; MAB1637; Millipore, Billerica, MA, USA). After extensively washing in PBS, cells were incubated with corresponding goat anti-mouse secondary antibodies conjugated to FITC (1:500; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and mouse anti-goat TRITC (1:500; Kirkegaard & Perry Laboratories), for 0.5 hours in the dark at room temperature, followed by extensive washes in PBS. Nuclei were stained with 10 ng/mL DAPI (Cat. No. A1001; Applichem, Darmstadt, Germany). Immunofluorescence was visualized with an inverted fluorescence microscope (Olympus, Tokyo, Japan).

Western blot assay

Astrocytes were infected with MSCV-Mash1 and MSCV retroviruses for 7 days, following which the protein expression of β-tubulin was analyzed. Infected cells were lysed with lysis buffer (P0013B; Beyotime, Shanghai, China) containing phenylmethyl sulfonylfluoride. Lysates were centrifuged at 12,000 × *g* at 4°C for 10 minutes. Protein concentrations were determined using BCA Protein Assay Kit (Cat. No. 23227; Pierce, Rockford, IL, USA). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane using a standard protocol. The membrane was incubated with the mouse primary antibody β-tubulin (1:1,000) and with secondary horseradish peroxidase-conjugated goat anti-mouse antibody (1:20,000; Cell Signaling Technology, Boston, MA, USA). The labeled proteins were visualized by chemiluminescence using enhanced chemiluminescence detection kit (Cat. No. 32106; Pierce).

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Author contributions: *Ding DF and Xu LQ had full access to experimental design, data collection, data analysis, and wrote the manuscript. Xu H, Li XF, Liang QQ, and Zhao YJ participated in some experiments and interpretation. Wang YJ was in charge of funding. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Peer review: *The characteristic of this study is that direct induction of reprogramming astrocytes into neurons can solve two problems: harvesting enough neural stem cells, and regulating the directional differentiation of transplanted cells. Authors found that Mash1 gene had the reprogramming effect on astrocytes into neuronal cells, which is innovative. Study results provided evidence for controlling the directional differentiation of neural cells, and were significant for translational medicine.*

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